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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

Seventh Quarterly Report of Progress

on

Research Project Number 4B04-14-004
Order Number FDO-5013

January 1 - March 30, 1962

Conducted by

Milk and Food Research, SEC

for the

U. S. Army Chemical Corps Biological Laboratories
Fort Detrick, Maryland

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U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio
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Seventh Quarterly Report of Progress

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**Research Project Number 4B04-14-004
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January 1 - March 30, 1962

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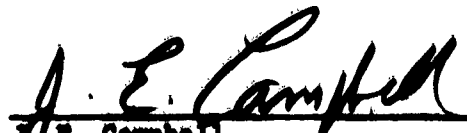
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ABSTRACT

Injection of paralytic shellfish poison (PSP) conjugated to formalin-treated bovine serum albumin (PSP-HCHO-BSA) into rabbits resulted in the production of sera capable of precipitating with their homologous antigen as well as with formalin treated heterologous protein. Anti-PSP-HCHO-BSA sera of high titer and strong reactivity were capable of protecting white mice from the lethal action of PSP when injected I.P. with serum and later challenged by the same route. Passive immunity was not conferred to mice which received sera that showed little or no ability to precipitate PSP-HCHO-BSA.

Evidence is presented to indicate that PSP does not exist in a stable protein conjugate in frozen Gonyaulax catenella cells.

CONTENTS

	<u>Page</u>
I. Introduction	1
II. Experimental	
Preparation of acid coupled conjugates of PSP and proteins	1
Studies on the chemical state of PSP in <u>Gonyaulax catenella</u> cells	8
III. Projected Research for Fourth Quarter, FY 1962	9
IV. Isolation and Purification of Paralytic Shellfish Poison	9
V. References	10
VI. Tables	11

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

I. Introduction

In the previous quarterly report (1) evidence was presented to indicate that a bi-phenyl bridged conjugate of paralytic shellfish poison and ovalbumin (PSP-azo-bi-phenyl-azo ovalbumin) elicited antibody responses in rabbits directed against the protein portion of the molecule. The PSP moiety did not appear to possess any haptenic properties in this system. In view of these findings this antigen system was abandoned in favor of studies on a conjugate of PSP and proteins coupled under acidic conditions. The experimental portion of this report includes a summary of the immunological and serological investigation of such a conjugate and some preliminary studies on the nature of paralytic shellfish poison as it exists in Gonyaulax catenella.

II. Experimental

Preparation of acid coupled conjugates of PSP and proteins.

A method of conjugating under acidic conditions PSP to formalinized bovine serum albumin (PSP-HCHO-BSA) was presented previously (1) along with some information on the reversible nature of the conjugate. Trial injections of these preparations stored at -10°C into 5-lb white rabbits resulted in death of the test animals and further suggested that the antigen had dissociated with the release of free poison.

For this reason the procedure previously described was slightly modified to minimize the toxicity of the conjugate.

A typical synthesis of five milliliters PSP-HCHO-BSA containing 2.5 mg BSA/ml and about 200 µg/ml of conjugated PSP was accomplished as follows: To 1.00 ml of protein solution containing 12.5 mg/ml of BSA in 0.15 M acetate buffer at pH 4.25 were added 0.13 ml of a five percent formalin and 0.075 ml of a PSP solution containing 19.2 mg/ml PSP by bioassay. The resulting solution was incubated at 26.5°C for 72 hours. The formalin concentration in the reaction mixture was 0.5 percent. At the end of the 72 hr. incubation period the reaction mixture was diluted to 5.0 ml and dialyzed at 25°C against 500 ml 0.001 M acetic acid (pH 3.9). The dialysis was continued until the dialysates, which were renewed at three-hour intervals, were non-toxic to mice. This required a total of six hours dialysis. The dialyzed antigen solution was then injected as quickly as possible.

The homologous control antigen, HCHO-BSA, was prepared as above to contain 2.5 mg BSA/ml but without the addition of PSP. Heterologous antigens of bovine β -lactoglobulin (BLG), PSP-HCHO-BLG, and HCHO-BLG, were also prepared in the same manner.

Four 5-lb white rabbits were immunized with each of the BSA antigens by intravenous injection into the lateral ear vein. The volume of the initial injections was 0.5 ml (1.25 mg protein); whereas subsequent injections were 1.0 ml (2.5 mg protein) in volume. An attempt was made to administer three injections per rabbit per week. This

schedule was adhered to, unless the rabbits displayed a weight loss, or the availability of a freshly prepared batch of antigen was delayed. In the event of weight loss, injections were not resumed until original weight was regained. Injections were given until each rabbit received a total of 26.25 mg of BSA protein. Seven days following the final injection, each rabbit was bled by cardiac puncture to obtain 30 to 40 ml of blood. The sera from these rabbits were stored in tubes in aliquots of 2 ml volumes at -10°C .

To titrate these sera, they were defrosted at room temperature and diluted 1:5 with physiological saline. The titration procedures were identical to those described in previous reports and the results are summarized in Tables I and II. These data reveal (Table I) that of four sera produced to PSP-HCHO-BSA antigen, two (9 and 11) sera displayed equivalently high titers to PSP-HCHO-BSA and HCHO-BSA antigens. Of the remaining two sera, serum 2 displayed no demonstrable precipitin content to either antigen, and serum 10 reacted only weakly with the two antigens. No demonstrable precipitin reaction to PSP was observed for any of the sera. The difference in the antibody levels of the four sera are probably related to variation in the immune response among the four rabbits.

The data presented in Table II reveal that of four sera produced to HCHO-BSA antigen only one (6) displayed high titer to its homologous antigen or to the PSP conjugated antigen. Serum 7 reacted weakly, and sera numbers 5 and 11 did not react.

In view of the response of sera 9, 10, and 11 to PSP-HCHO-BSA antigen, an attempt was made to inhibit this reaction by the introduction of PSP to the system (hapten inhibition test). The procedures for the inhibition tests were identical to those described in previous reports and the results are summarized in Table III. The data indicate that no inhibition of the reaction between PSP-HCHO-BSA and anti-sera specific for it occurred at any of the concentrations of poison or serum dilutions employed. In view of the concentration of histamine required to inhibit a hapamine-anti hapamine system (See First Quarterly Report of Progress, July to September, 1960), the results shown in Table III are not surprising. A minimum of 10 mg of histamine was found necessary to completely inhibit the hapamine-anti-hapamine system. Milligram quantities of PSP may be inhibitory to the system presently under study; but, due to the need to conserve the poison supply, inhibition studies employing such quantities were not undertaken.

An alternative procedure to expending large quantities of poison in inhibition tests, was to tritrate the PSP-HCHO-BSA antibody against a heterologous antigen. A reaction between anti PSP-HCHO-BSA sera and PSP-HCHO-BLG would indicate that the PSP moiety of the formalin treated BSA conjugated antigen was capable of reacting as a hapten. The results of such an experiment along with appropriate controls are presented in Tables IV and V.

The data in Table IV reveal that a precipitin reaction occurred between PSP-HCHO-BSA antisera and PSP-HCHO-BLG. The same reaction was

noted to HCHO-BLG, indicating that the cross reaction in the PSP-containing systems was not due to the action of PSP as a hapten, but to the occurrence of a serologically related material in the formalinized proteins. Landsteiner(2,3), Horsfall(4), and Jacobs and Sommers(5) reported that antibodies produced to formalinized sera precipitated with formalinized sera from serologically unrelated species, although less intensely than with homologous native or formalinized serum. The results of these workers indicate that formalinization of proteins attributes to them an additional new and characteristic specificity with little or no attendant loss of species specificity.

To determine that the "nonspecific" reactions recorded in Table IV were due to the occurrence of a serologically related material resulting from formalin treatment of the proteins, a similar titration was performed in which native BLG was included in addition to formalinized BLG. As a further precaution, to denote the extent to which residual traces of free formalin might contribute to denaturation and non-specific precipitation, normal rabbit serum was included as one of the test sera. The results of these titrations are shown in Table V. These data confirm the findings shown in Table IV and those of the other workers cited above in that the antibodies produced to PSP-HCHO-BSA precipitated with the heterologous formalin-treated protein. The unexpected result of this experiment was the reaction of the immune sera to heterologous native BLG. The weak nature of this reaction and the fact that both proteins were of a bovine source leads to the conclusion that

trace amounts of BSA may have been present in the BLG preparation. The absence of reaction between normal rabbit serum and native and formalin-treated proteins eliminates from the above results the possibility of a "chemical precipitation", due to denaturation of serum proteins by formalin.

In view of the number of precipitating antibody systems apparently present in the sera produced to PSP-HCHO-BSA, additional serological work with these sera will necessarily be restricted to agar diffusion techniques in an attempt to separate and identify the antibodies.

In addition to determining the precipitating antibodies present in PSP-HCHO-BSA antisera, preliminary investigations were made of the ability of these sera to confer passive immunity to mice. White mice (Hamilton Laboratories) weighing between 19 and 22 grams were employed. The mice were divided into groups of two mice each. The mice in each group received 2.0 ml of undiluted anti PSP-HCHO-BSA sera as two 1.0 ml I.P. injections given 24 hours and 4 hours, respectively, prior to challenge. The protected mice were challenged with 1.0 ml of an aqueous solution of purified PSP containing 0.3 micrograms per ml. For use as controls, an additional group of mice received normal rabbit serum and another group received sterile distilled water. The results of this first protection test are shown in Table VI. These data reveal that mice protected with 2.0 ml of undiluted, high-titered, anti PSP-HCHO-BSA sera (9 and 11) did not die; whereas mice which received similar quantities of undiluted normal serum (1) or distilled water

were not protected. The slightly extended death times for the mice receiving normal serum over those for the mice which received water is probably related to the retention of residual amounts of serum in the intraperitoneal cavity which may interfere with the normal rate of absorption of poison. The immune sera employed in this test were numbers 9 and 11 which were shown to yield good precipitation reactions (See Table I) to their homologous antigen. In view of the possible correlation between high precipitin titer and neutralizing power of the sera, the experiment was repeated employing serum 9 (high titer) and sera numbers 2 and 10. Serum 2 displayed no precipitin titer and serum 10 reacted only weakly to PSP-HCHO-BSA (Table I).

In addition, a different normal rabbit serum (4) was employed. The results of this experiment are given in Table VII. Once again it was noted that mice receiving serum 9 (high titer) were protected. Mice which received normal rabbit serum or distilled water died. The interesting observation noted from this experiment was that mice which received non-precipitating serum (2) or weakly-precipitating serum (10) also died.

The results of these neutralization tests indicate that antibodies capable of neutralizing PSP are produced in rabbits injected with PSP conjugated to formalin treated BSA; that these antibodies may be transferred to mice to yield passive immunity; and that a correlation may exist between the precipitating power of a serum and its ability to neutralize PSP.

Studies on the chemical state of PSP in Gonyaulax catenella cells.

Studies were initiated on the chemical state of PSP in the frozen G. catenella cells received January 30, 1962, from Dr. Dudley Glick. The toxicity of an acid hydrolysate of these cells showed that they contain 198 µg PSP per gram of frozen cells. It was of particular interest both from the immunological and chemical viewpoint to determine whether the PSP exists in the cells in a stable protein conjugated form. It was anticipated that studies on the dialyzability of the PSP contained in the cells should provide useful information. Accordingly, a weighed sample of the frozen cells was dialyzed in a physiological saline medium with vigorous agitation over a period of three days at room temperature. Toxicity data on the dialysates and on an acid hydrolysate of the cellular material remaining within the dialysis bag showed that only the dialysates contained PSP. In order to minimize cell wall rupture, another weighed sample of frozen cells was dialyzed without agitation and in a medium having the ionic composition of sea water. When the toxicity data were corrected for the presence of high salt concentrations according to the findings of Schantz et al.⁽⁶⁾, the dialysates were found to contain 85-90% of the PSP known to be present in the cells. An acid hydrolysate of the cellophane bag contents was non-toxic. These findings indicate that PSP does not exist as a stable protein conjugate in the frozen cells.

III. Projected Research for Fourth Quarter, FY 1962

In view of the promising results from the initial investigation of the PSP-HCHO-BSA reactions primary attention will be given to the confirmation and extension of these findings. Efforts will be made to (a) increase the antibody titers of sera to PSP-HCHO-BSA by hyperimmunization of previously immunized rabbits, (b) investigate the protective capacities of sera on hand and those to be obtained by hyperimmunization by means of serial titrations in mice to determine the ratio of serum to poison required for neutralization, (c) to separate and identify by means of agar diffusion techniques the various antibody systems present in anti PSP-HCHO-BSA sera, and (d) investigate alternate serological methods suitable for rapid in vitro demonstration of poison by means of these sera.

IV. Isolation and Purification of Paralytic Shellfish Poison

Arrangements have been made for Mr. E. F. McFarren, Project Officer for this phase of the work, to visit the State of Alaska Department of Health and Welfare. He will confer with Mr. Amos Alter, Mr. Alfred Baker and others who will be involved in the collection and shipment of the toxic clam siphons to ascertain any specific problems they might have and to facilitate getting this work underway this spring. It is anticipated that the first batches of clam siphons will arrive in Cincinnati during the latter part of April. Processing will begin

immediately on arrival. All the equipment and space necessary for this operation are now available.

V. References

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Table I.
Titration of Anti PSP-HCHO-BSA Sera with Various Antigens

Serum No.*	Antigens 0.4 ml per tube	Dilutions of Antigens									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
2	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	-
	HCHO-BSA	-	-	-	-	-	-	-	-	-	-
	PSP	-	-	-	-	-	-	-	-	-	-
9	PSP-HCHO-BSA	-	2+	3+	4+	4+	4+	3+	2+	2+	+
	HCHO-BSA	+	2+	3+	4+	4+	4+	3+	2+	+	+
	PSP	-	-	-	-	-	-	-	-	-	-
10	PSP-HCHO-BSA	-	-	-	+	+	+	2+	+	±	-
	HCHO-BSA	-	-	-	-	-	±	+	+	±	±
	PSP	-	-	-	-	-	-	-	-	-	-
11	PSP-HCHO-BSA	±	±	±	3+	3+	4+	4+	3+	2+	+
	HCHO-BSA	+	+	2+	3+	3+	4+	4+	3+	2+	+
	PSP	-	-	-	-	-	-	-	-	-	-

Serum-saline controls negative
Antigen-saline controls negative
*Serum diluted 1:5, 0.4 ml per tube

Table II
Titration of Anti HCHO-BSA Sera with Various Antigens

Serum No.*	Antigens 0.4 ml per tube	Dilutions of antigens									
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	
5	HCHO-BSA		-	-	-	-	-	-	-	-	
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	
6	HCHO-BSA		+	2+	2+	3+	4+	3+	2+	+	
	PSP-HCHO-BSA	±	+	2+	3+	4+	3+	+	+	±	
7	HCHO-BSA		-	-	-	-	±	+	±	±	
	PSP-HCHO-BSA	-	-	-	-	±	±	+	±	±	
8	HCHO-BSA		-	-	-	-	-	-	-	-	
	PSP-HCHO-BSA	-	-	-	-	-	-	-	-	-	

Serum-saline controls negative
Antigen-saline controls negative
*Serum diluted 1:5, 0.4 ml per tube

Table III

Inhibition of Reaction Between Anti PSP-HCHO-BSA Sera and PSP-HCHO-BSA Antigen by PSP

Serum No.	Antibody and dilution 0.2 ml per tube	Antigen and dilution 0.4 ml per tube	Saline	Concentration of PSP per tube in micrograms									
				172.8	86.4	43.2	21.6	10.3	5.15	2.575	1.2875	0.64375	
9	Anti PSP-HCHO-BSA 1:2.5	PSP-HCHO-BSA 1:128	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Anti PSP-HCHO-BSA 1:5	PSP-HCHO-BSA 1:128	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Anti PSP-HCHO-BSA 1:10	PSP-HCHO-BSA 1:128	+	+	+	+	+	+	+	+	+	+	+
10	Anti PSP-HCHO-BSA 1:2.5	PSP-HCHO-BSA 1:128	+	+	+	+	+	+	+	+	+	+	+
	Anti PSP-HCHO-BSA 1:5	PSP-HCHO-BSA 1:128	-	-	-	-	-	-	-	-	-	-	-
	Anti PSP-HCHO-BSA 1:10	PSP-HCHO-BSA 1:128	-	-	-	-	-	-	-	-	-	-	-
11	Anti PSP-HCHO-BSA 1:2.5	PSP-HCHO-BSA 1:128	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Anti PSP-HCHO-BSA 1:5	PSP-HCHO-BSA 1:128	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	Anti PSP-HCHO-BSA 1:10	PSP-HCHO-BSA 1:128	+	+	+	+	+	+	+	+	+	+	+

Serum-saline controls negative
Antigen-saline controls negative

Table IV
Reactions of Anti-PSP-HCHO-BSA Sera to PSP-HCHO-BLG Antigen

Serum No.	Antibody 1:5 dilution 0.4 ml per tube	Antigen 0.4 ml per tube	Dilutions of Antigens									
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
9	Anti PSP-HCHO-BSA	PSP-HCHO-BLG	4+	4+	4+	3+	2+	+	-	-	-	-
10	Anti PSP-HCHO-BSA	PSP-HCHO-BLG	-	-	-	-	-	-	-	-	-	-
11	Anti PSP-HCHO-BSA	PSP-HCHO-BLG	4+	4+	4+	3+	2+	2+	+	-	-	-
9	Anti PSP-HCHO-BSA	HCHO-BLG	4+	4+	4+	3+	2+	+	+	-	-	-
10	Anti PSP-HCHO-BSA	HCHO-BLG	-	-	-	-	-	-	-	-	-	-
11	Anti PSP-HCHO-BSA	HCHO-BLG	4+	4+	4+	3+	2+	+	+	-	-	-

Serum-saline controls negative.
Antigen-saline controls negative.

Table V
Reactions of Anti PSP-HCHO-BSA Sera to Various BLG Antigens

Serum No.	Antibody	Antigen 0.4 ml per tube	Dilutions of Antigens							
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
9	Anti PSP-HCHO-BSA	PSP-HCHO-BLG	+	+	3+	3+	+	-	-	-
	Anti PSP-HCHO-BSA	HCHO-BLG	+	+	2+	3+	2+	2+	+	-
	Anti PSP-HCHO-BSA	BLG	+	+	2+	+	+	±	-	-
11	Anti PSP-HCHO-BSA	PSP-HCHO-BLG	+	+	2+	+	+	±	-	-
	Anti PSP-HCHO-BSA	HCHO-BLG	2+	2+	3+	2+	+	+	±	-
	Anti PSP-HCHO-BSA	BLG	±	2+	2+	+	+	+	±	-
1	Normal rabbit serum	PSP-HCHO-BLG	-	-	-	-	-	-	-	-
	Normal rabbit serum	HCHO-BLG	-	-	-	-	-	-	-	-
	Normal rabbit serum	BLG	-	-	-	-	-	-	-	-

Serum-saline controls negative
Antigen-saline controls negative

Table VI

Passive Immunization of White Mice to PSP by Anti PSP-HCHO-BSA Sera

Mouse No.	Weight of mouse in grams	Number and type of undiluted serum received I.P.	Hours before challenge serum received	Volume of undiluted serum received I.P.	Micrograms PSP/ml (1.0 ml challenge) I.P.	Time of death (minutes)
1	20.80	Rabbit 11	24	1.0 ml.	0.3	No death
		Anti PSP-HCHO-BSA	4	1.0 ml.		
2	19.45	Rabbit 11	24	1.0 ml.	0.3	No death
		Anti-PSP-HCHO-BSA	4	1.0 ml.		
3	20.38	Rabbit 9	24	1.0 ml.	0.3	No death
		Anti PSP-HCHO-BSA	4	1.0 ml.		
4	22.00	Rabbit 9	24	1.0 ml.	0.3	No death
		Anti PSP-HCHO-BSA	4	1.0 ml.		
5	21.40	Rabbit 1	24	1.0 ml.	0.3	7 min. 20 sec.
		Normal serum	4	1.0 ml.		
6	19.18	Rabbit 1	24	1.0 ml.	0.3	6 min. 35 sec.
		Normal serum	4	1.0 ml.		
7	20.58	Distilled water	24	1.0 ml	0.3	6 min. 15 sec.
			4	1.0 ml.		
8	20.87	Distilled water	24	1.0 ml.	0.3	5 min. 17 sec.
			4	1.0 ml.		

Table VII

Passive Immunization of White Mice to PSP by Anti PSP-HCHO-BSA Sera

Mouse No.	Weight of mouse in grams	Number and type of undiluted serum received	Hours before challenge serum received	Volume of undiluted serum received I.P.	Micrograms PSP/ml. (1.0 ml challenge) I.P.	Time of death (minutes)
1	20.78	Rabbit 2*	24	1.0 ml	0.3	7 min 13 sec
		Anti PSP-HCHO-BSA	4	1.0 ml		
2	20.13	Rabbit 2*	24	1.0 ml	0.3	8 min 20 sec
		Anti PSP-HCHO-BSA	4	1.0 ml		
3	20.11	Rabbit 9	24	1.0 ml	0.3	No death
		Anti PSP-HCHO-BSA	4	1.0 ml		
4	21.66	Rabbit 9	24	1.0 ml	0.3	No death
		Anti PSP-HCHO-BSA	4	1.0 ml		
5	19.51	Rabbit 10**	24	1.0 ml	0.3	7 min 14 sec
		Anti PSP-HCHO-BSA	4	1.0 ml		
6	20.95	Rabbit 10**	24	1.0 ml	0.3	7 min 43 sec
		Anti PSP-HCHO-BSA	4	1.0 ml		
7	21.40	Rabbit 4	24	1.0 ml	0.3	11 min 40 sec
		Normal serum	4	1.0 ml		
8	21.00	Rabbit 4	24	1.0 ml	0.3	8 min 52 sec
		Normal serum	4	1.0 ml		
9	20.96	Distilled H ₂ O	24	1.0 ml	0.3	5 min 20 sec
			4	1.0 ml		
10	20.46	Distilled H ₂ O	24	1.0 ml	0.3	4 min 55 sec
			4	1.0 ml		

*Anti PSP-HCHO-BSA serum from rabbit #2 contained no demonstrable precipitating antibodies to homologous antigen.

**Anti PSP-HCHO-BSA serum from rabbit #10 reacted weakly with its homologous antigen.